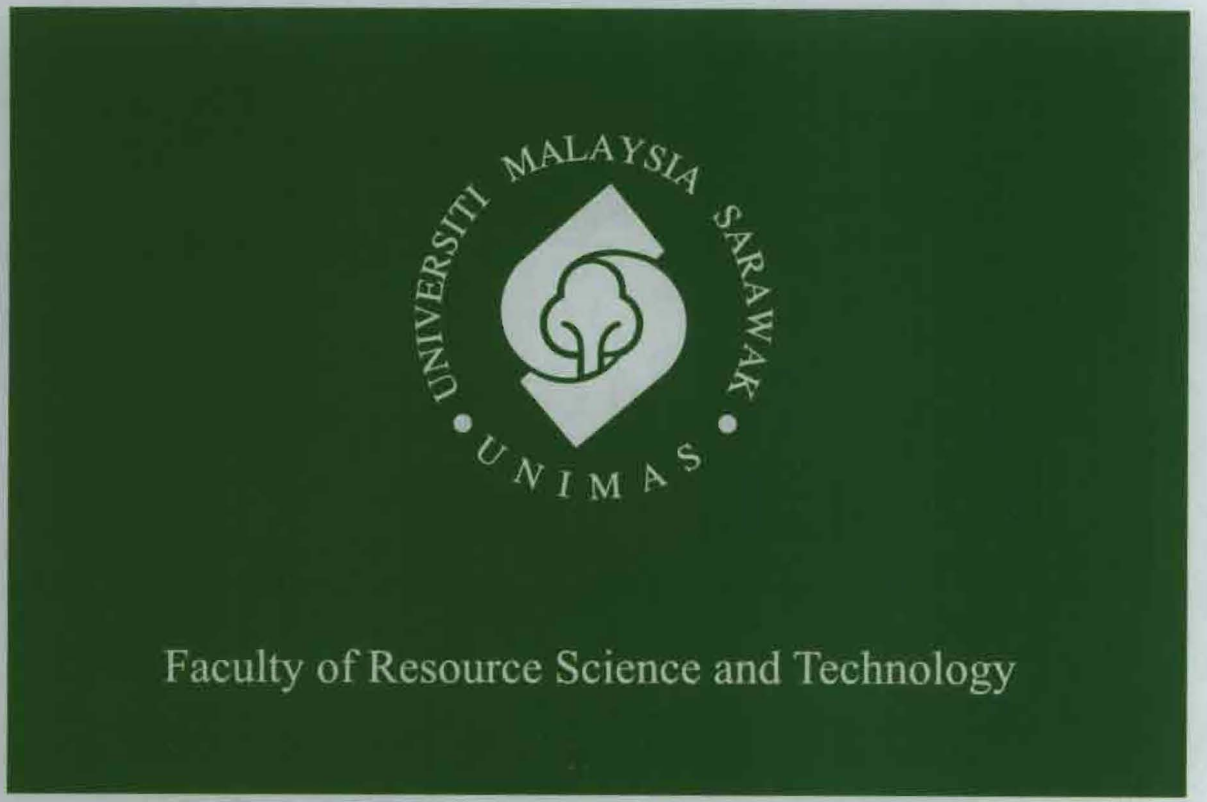

ISOLATION AND CLONING OF *ABCA2* GENE FROM *RASBORA SARAWAKENSIS*

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Bachelor of Science with Honours
(Resource Biotechnology)
2015

Isolation and Cloning of *ABCA2* Gene from *Rasbora Sarawakensis*

Tan Say Yen (39046)

A thesis submitted in partial fulfillment of the Final Year Project 2(STF 3015) Resource Biotechnology

Supervisor: Dr. Chung Hung Hui

Resource Biotechnology
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12/5/2015

Acknowledgement

First and foremost, I would like to express my sincere gratitude and appreciation to my project supervisor, Dr. Chung Hung Hui for his guidance and support throughout the whole period of my final year research. It has been an honour for me to work on this project under his supervision. He shared his valuable experiences, brilliant suggestion and advice throughout the whole project.

Besides, I would also like to thank my fellow lecturers for providing the necessary equipments and agents in this project, especially Dr. Lee Kui Soon, Assoc. Prof. Dr. Edmund Sim Ui Hang, Assoc. Prof. Dr. Mohd Hasnain Md Hussain, and Dr. Lesley Maurice Bilung. I would like to extend my gratitude to post-graduate students in the Molecular Biology Department especially Shek Li and Shamil who willing to give advice and guidance in order for me to overcome the problems during my research. Without them, this project would not have been completed.

I am extremely thanks to my labmates in Animal Biotechnology Laboratory, especially Annie, Fazli, Azllan, Aimi, dan Qawiem, who always give mental support and assist me in the period of my research.

I am blessed to have my family support through all the decision that I make in life. Their selflessness and believe in providing me in the way to the success of future. Their supports help me to achieve my full potential for which I will be indebted for life.

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DECLARATION OF ORIGINAL WORK

This declaration is made on the 22 day of June 2015.

Student's Declaration:

I, Tan Say Yen, 39046, Faculty of Resource Science and Technologyhereby declare that the work entitled Isolation and Cloning of *ABCA2* Gene from *Rasbora Sarawakensis*is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

22 - 6 - 2015
Date submitted

Tan Say Yen (39046)

Supervisor's Declaration:

I, Dr Chung Hung Hui hereby certifies that the work entitled Isolation and Cloning of *ABCA2* Gene from *Rasbora Sarawakensis* was prepared by the above named student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment ofBachelor of Science with Honours, and the aforementioned work, to the best of my knowledge, is the said student's work.

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List of Abbreviations

ABC	ATP-Binding Cassette
ABCP	Placenta-specific ABC Protein
ATP	Adenosine Triphosphate
BCRP	Breast Resistance Associated Protein
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
DNA	Deoxyribonucleic Acid
EST	Expressed Sequence Tag
GSH	Glutathione
HDL	High Density Lipoprotein
MDR1	Multi-Drug Resistance Gene
mRNA	Messenger Ribonucleic Acid
MRP	Multidrug Resistance-associated Proteins
MXR	Mitoxantrone-Resistance Protein
NBD	Nucleotide-Binding Domain
NCBI	National Center for Biotechnology Information
NTP	Nucleoside Triphosphate
PCR	Polymerase Chain Reaction
PFIC	Progressive Familial Intrahepatic Cholestasis
Pgp	P-glycoprotein
PXE	PseudoxanthomaElasticum
rRNA	Ribosomal Ribonucleic Acid
TMD	Transmembrane Domain
UV	Ultraviolet

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Isolation and Cloning of *ABCA2* Gene from *Rasbora Sarawakensis*

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Abstract

ABC genes encode ABC transporter protein that plays an important role in ATP hydrolysis in which to assist the solute to cross the plasma membrane. Mutation of ABC genes lead to human diseases such as Alzheimer, PFIC, and PXE. The aim of this research is to identify the expression of *ABCA2* gene in *Rasbora sarawakensis* and subsequently clone into pGEM-T® Easy Vector. Total RNA was initially isolated from whole fish homogenate using Tri reagent and phenol chloroform precipitation. First strand cDNA were generated and *ABCA2* transcript was amplified with PCR using degenerate primers. A 464 bp amplicon band yielded was then gel extracted and further cloned into vector. Transformation using in house prepared *Escherischia coli* XL1 yielded an efficiency of 10^6 transformants, with 60 of blue and 8 of white colonies. Subsequently, four white colonies were verified with colony PCR and only one was showed the presence of insert. Further verification of insert using *NotI* restriction digestion was conducted which yielded two discreet bands. The plasmid minipreparation product was then sent for sequencing and the result was verified using BLAST. BLAST analysis registered an E-value of 2e-180 with highest similarity to *Danio rerioABCA2* gene. Based on this study, further temporal and spatial expression of *ABCA2* gene should be well and further study in order to fully utilize *R. sarawakensis* as a model organism for disease study.
Keywords: ABC transporter, *ABCA2*, PCR, *Rasbora sarawakensis.*

Abstrak

ABC gen mengekod ABC pengangkut protein yang memainkan peranan penting dalam ATP hidrolisis yang membantu dalam pengangkutan membran plasma. Mutasi gen ABC menyebabkan penyakit seperti Alzheimer, PFIC, dan PXE. Tujuan kajian ini adalah untuk mengenal pasti ekspresi gen ABCA2 dalam Rasbora sarawakensis dan seterusny mengklon ke dalam pGEM-T® Easy Vektor. Keseluruhan RNA pada mulanya diasingkan daripada ikan homogenate dengan menggunakan Tri reagen dan pemendakan fenol kloroform. Sintesis pertama helai cDNA telah dihasilkan dan ABCA2 transkrip telah digandakan menggunkan PCR dengan degenerat primer. 450 bp amplicon telah dihasilkan kemudian diekstrak daripada gel dan diklonkan ke dalam vektor. Transformasi menggunakan Escherischia coli XL1 yang disediakan sendiri telah menghasilkan transformants efisien 10^6 dengan 60 koloni biru and 8 koloni putih. Empat koloni putih telah disahkan dengan koloni PCR dan hanya satu yang menunjukkan keputusan yang positif. Kemudiannya, verifikasi lanjut menggunakan sekatan penghadaman NotI untuk menghasilkan dua spesifik band. Produk plasmid minipreparation kemudian dihantar untuk penjujukan dan keputusan yang telah disahkan menggunakan BLAST. Analisis BLAST mencatatkan E-nilai 2e-180 mencapai persamaan tertinggi semasa dibandingkan dengan gen ABCA2 dalam Danio rerio. Berdasarkan kajian ini, ekspresi temporal dan spatial gen ABCA2 boleh dikenalpasti dan kajian lanjut boleh dilakukan untuk menggunakan sepenuhnya R. sarawakensis sebagai model organisma untuk kajian penyakit.
Kata kunci: ABC pengangkut, ABCA2, PCR, Rasbora sarawakensis.

1.0 Introduction

1.1 Background

A cytoplasmic membrane acts as a barrier for cell to separate the interior of the cell from their external environment which allow certain essential ions, intermediate metabolite, proteins and other components remain within the cell. Therefore, transport systems have been developed during the evolution to ensure that the ions and metabolic intermediates are entering the cell and other compounds are left external to the cell (Jasinski et al., 2003). ATP-binding cassette (ABC) transporters are one of the transport systems involved in such as process. They are one of the largest protein families in transporter that can be categorized into classes, families and subfamilies based on the phylogenetic analyses.

Human genome consists of 49 ABC genes that are divided into seven subfamilies termed as ABCA to ABCG (Vasiliou et al., 2009). Each of these diverse transporter families has members that take part in different roles in many cellular processes. For examples in subfamily A of the ABC family (ABCA) contains 12 genes that are involved mostly in lipid trafficking in many different organs and cell types (Vasiliou et al., 2009). ABCA subfamily consists of large transporter proteins with the largest transporter detected having 2100 amino acids.

To date, there are more than 11 ABC genes that are related to human genetically inherited diseases in mutation. The mutated *ABCA1* gene might lead to Tangier disease T1 due to the defective apolipoprotein-I-induced lipid outflow (Walter et al., 2004). This happen because the amount of high density lipoprotein (HDL) are reduced dramatically in most of the affected organism. However, *ABCA2* may be involved in brain sterol homeostasis which is highly associated with early stages of the Alzheimer's disease and might lead to its neural expression (Broccardo et al., 2006). Based on the previous finding,

ABCA2 is found in the intracellular vesicles that have been identified in the lysosome-related organelles. It acts as a marker of neural progenitors (Broccardo et al., 2006) in the development of the adult rodent brain.

In my research, *Rasbora sarawakensis* is chosen as my model organism because it is an endemic freshwater fish species that can be found in Asia. It can be harvested easily in Sungai Sarawak and Batang Kayan in Sarawak and also Mempawah and Malawi in Kalimatan Barat. *R. sarawakensis* is a teleost which is under the same family Cyprinidae with the famous model organism, *Danio rerio.* According to Liao (2010), they found that there are the interrelationships among the large genus Rasbora species between Asian and African genera of Danioninae as well as the relationship with other danionines genus in term of analyzing the morphological and DNA data.

Based on previous research findings, it is well-acknowledged that *ABCA2* gene is essentially taking part in the ATP catabolic process. However, there is currently a lack of information on *R. sarawakensisABCA2* expression whereby embryonic research provides a further clue on how localization of transcript may affect tissue development. Therefore, in order to improve the understanding of *ABCA2*, the development of targeted cell and tissue, such as brain, kidney and liver will be focused on *R. sarawakensis* by using RNA expression analysis technique.

1.2 Objectives

The aims of this project are:

1. To isolate the *ABCA2* transcript from*R. sarawakensis*.
2. To clone a partial fragment of *ABCA2* gene from *R. sarawakensis*.

2.0 Literature review

2.1 Membrane transport proteins

Membrane proteins are the important components that are built up in the cell membrane with phospholipid bilayer. The function of the membrane transport protein is to transfer different polar molecules such as ions, macromolecules like sugars, amino acids, nucleotides and other cell metabolites (Alberts et al., 2002). Membrane transport proteins are divided into two major classes, i.e. carrier proteins and channel proteins. Carrier proteins allow only certain specific solute to bind on the transporters and transport the solute across the plasma membrane by undergoing conformational changes (Alberts et al., 2002). However, channel protein in the form aqueous pore bind to the transporter rather than carrier proteins. This is because they usually involved very weak interaction between solute and channel protein. The channel proteins allow the extension of the phospholipid bilayer to open these aqueous pores and then allowing specific solutes such as inorganic ions to pass through these pores (Alberts et al., 2002).

Carrier proteins, such as transporter's function are to facilitate the movement of a specific substrate by binding only one or a few of substrate molecules at once (Lodish et al., 2000). The transporter undergoes conformational change and transport the substrate across the plasma membrane after the substrate is bound at the specific site on the transporter. Since this type of transport system require the conformation change of the transporter, the movement of molecules becomes slower in which the transporters move only about 10^2 – 10^4 molecules in a second (Lodish et al., 2000).

Furthermore, ATP-power pumps are the membrane transporter proteins that use the energy released by ATP hydrolysis to carry the substrates across the membranes. The direction of molecules movement is against a chemical concentration gradient or electric

potential (Lodish et al., 2000; Vasiliou et al., 2009). In addition, ABC transporters can be divided into two i.e. importers or exporters based on the direction of transport relative to the cytoplasm (Vasiliou et al., 2009).

2.2 The superfamily of ABC efflux transport

One of the largest families of transmembrane transporter proteins is the ABC gene superfamily. The ABC genes are categorized into seven subfamilies in mammal which are ABCA to ABCG based on the percentage of amino acid identity. Currently, there is one extra subfamily existed only in fish species which is *ABCH* gene (Lončar et al., 2010; Ferreira et al., 2014). To date, some of the ABC transporter superfamily has been characterized from mammal and there are ongoing researches into the important roles of each subfamily (Jeong et al., 2014). A total of 58 members of the ABC family have been illustrated in various species and 49 out of total members were found in human genome. The remaining gene was found in other animal species (Ferreira et al., 2014).

The first and best illustrated ABC transporter is P-glycoprotein (Pgp) which came from subfamily B member 1. Pgp is encoded by *MDR1* gene whereby it takes part in drug resistance. Based on the previous finding, drug accumulation would be increased in *ABCB1* gene knockout organisms compare to the wild-type organism and decreased drug accumulation with its MDR abilities when the cell transfected with *ABCB1* gene (Ferreire et al.,2014).

Next, *ABCC1* was discovered after the discovery of *ABCB1*. ABC subfamily C consists of 13 members in which most of them are active ATP-dependent membrane transporters for organic negative charge ions of therapeutic substances (Ferreire et al., 2014). This group of subfamily involved in mediating drug resistance in which play important role in organ defense.

Furthermore, one of the subfamily highly involved in anticancer drugs of transportation is the second member of the ABCG subfamily (*ABCG2*) and they are contributing to a MDR phenotype. According to Ferreira et al. (2014), this protein could be breast resistance associated protein (BCRP), mitoxantrone-resistance protein (MXR) or placenta-specific ABC protein (ABCP). Overexpression of *ABCG* gene in long term cell-lines has been correlated with high resistance levels to several of anticancer drugs. Moreover, *ABCG2* has higher affinity to transport a wide range of substrates form chemotherapeutic agents to organic negative charge ions. For example, the transportation of sulfated conjugates of steroids and xencbiotics over glucoronide and GSH metabolites occurred in *ABCG2* gene (Ferreira et al., 2014).

Discovery of the important of the ABC efflux transporters in mammal could be proposed that a similar mechanism may also be happened in aquatic organism. According to Lončar et al. (2010), more than 40 aquatic species was investigated to characterize the Pgp and MRP types of efflux transporters.

2.3 Mechanism for ABC transporters

ATP-binding cassette (ABC) transporters are made of ATPase domains which also known as nucleotide-binding domains (NBDs) that are involved in the ATP hydrolysis which is catalyzed by cytoplasmic ABCs. They hydrolyzed ATP to provide energy for the transport of substrates in and out of the cytoplasm through phospholipids bilayer by using importers and exporters. On the other hand, transmembrane domains (TMDs) facilitate the substrate for translocation (Locher, 2008) when a substrate binding protein is present. The Figure 2.1 shows the schematic of ABC transporter function that is involved the importers and exporters.

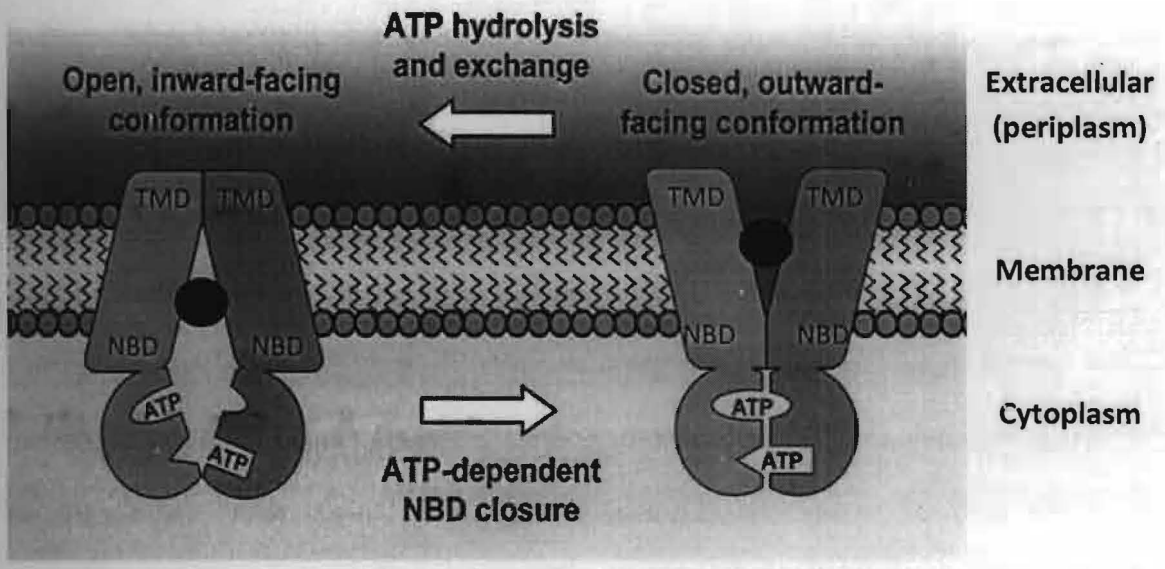

Figure 2.1 Mechanism of an ABC transporter. ATP dependent closure/ dimerization of cytosolic NBDs provides energy that pulls the TMDs from open to closed conformation. (Adapted from Procko et al., 2009)

NBDs contain two sub-domains which are functionally unrelated RecA protein and helical sub-domain. In additions, NBDs also consists of several important motifs such as P-loops and LSGGQ whereby P-loops motif is located in the RecA-like sub-domain and the LSGGQ motif is located in the helical sub-domain (Locher, 2008). To form a complete transporter, two NBDs are arranged adjacent along the plasma membrane in which these motifs are exhibited on the fragment surface in a head-to-tail arrangement to provide a binding site to ATPsduring ATP hydrolysis (Figure 2.2).

In the absence of a nucleotide, the open at the domain surface can be accessed with the water at the nucleotide-binding sites. However, when the ATP is bound, the surface closes and the nucleotides are squeezed tightly between the NBDs (Chen et al., 2003). If one of the NBDs is mutated, the ATP hydrolysis by these transporters is prevented, for example in the cystic fibrosis transmembrane conductance regulator (CFTR).

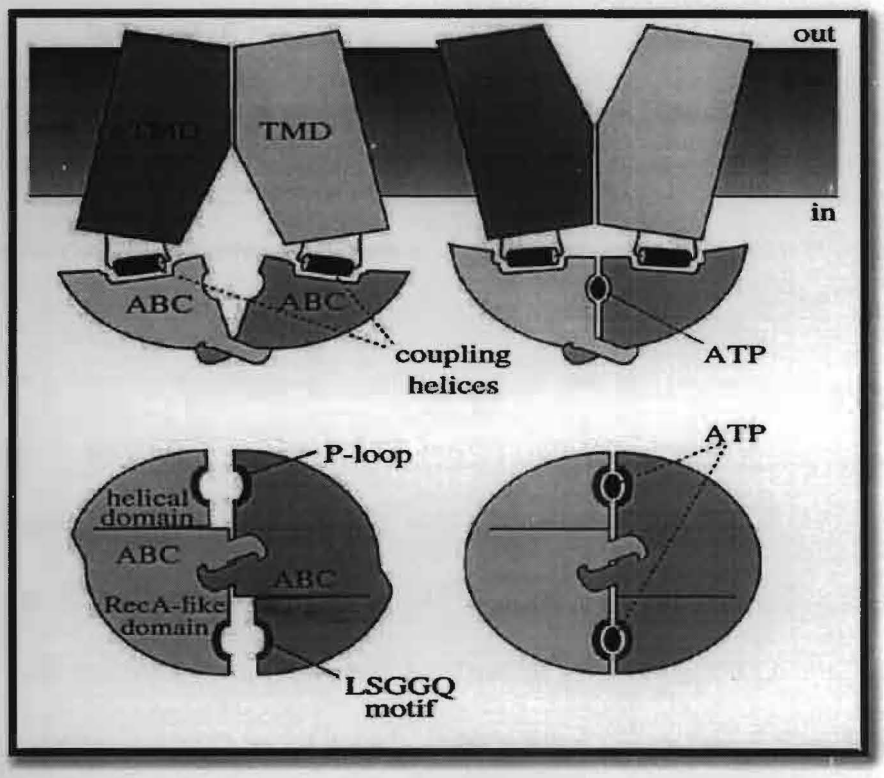

Figure 2.2 Conserved coupling mechanism of ABC transporters. (Adapted from Locher, 2008)

2.4 *ABCA2* gene

ABCA2 is a gene that encodes ATP-binding cassette transporter member 2 in subfamily A protein whereby it is located at 3p13 on chromosome 3 (Figure 2.3) in Norway rats. The length of mRNA of *ABCA2* is 8040 bp with accession number of NM_024396 which had first been published by Zhao et al. (2000). The coding sequence begins from the ±68 bp to ±7372 bp of the complete mRNA length which consists of 2434 amino acids. Furthermore, the transcript also contains 49 exons and 51 introns in the Norway rat.

For higher level of vertebrate organism such as human, *ABCA2* is also found on human chromosome 9 which is located at 9q34 (Figure 2.4) with 50 exons and 49 introns. The length of the transcript is between 8163 bp and 8171 bp since there are two alternative transcripts occurring within the 2436 amino acids based on Kaminski et al. (2001). According to Zhao et al. (2000) finding, the ABCA protein of 2434 amino acid in rat has

found a range of 40% to 44.5% identity with ABCA protein in mouse and human respectively.

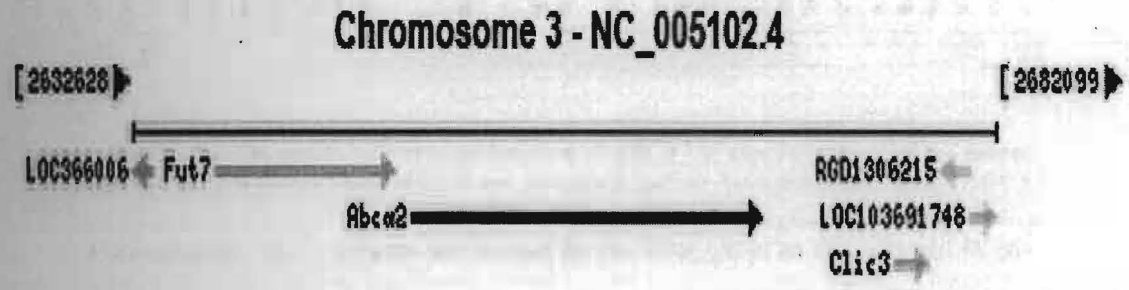

Figure 2.3 The longest arrow indicates the location of *ABCA2* in chromosome 3 of the Norway rat. (Adapted from NCBI, http://www.ncbi.nlm.nih.gov/gene/79248)

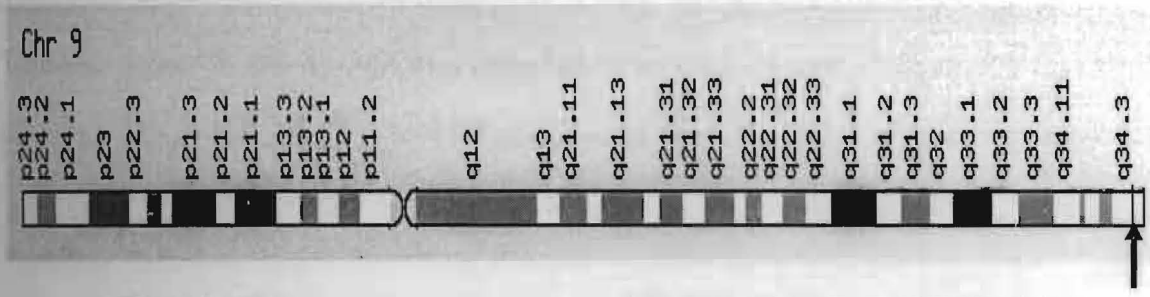

Figure 2.4 The arrow indicates the location of *ABCA2* in chromosome 9 of the human. (Adapted from GeneCards, http://www.genecards.org/cgi-bin/carddisp.pl?gene=ABCA2)

Furthermore, two variants are found in the first exon to the second in the transcript during alternative splicing which are termed as 1A and 1B. The alternative splicing is caused by the presence of unique amino terminus in a protein (Ile et al., 2004) where it forms an exon 1B which contains coding sequence for 52 amino acids whereas 22 amino acids for exon 1A (Ile et al., 2004). Both variants are involved with lysosome-associated membrane proteins and they are also more likely to be functionally redundant.

2.5 Cyprinidae family

The biggest family of freshwater fish is Cyprinidae which contains approximately 2420 species in estimated 220 genera (Nelson, 2006). The large scale of the data is required to group this family according to its geographic distribution, morphological, anatomical characteristics, and its species (Wang et al., 2012). They are highly variable in

term of morphology which depended on their diversified habitat. In addition, Cyprinidae family may showed the evolutionary rates of the trait they adapted and differentiating between the convergences and traits they shared from the common ancestry (Wang et al., 2012).

There are two main lineages, the cyprinine and the leuciscine groups in which they can distinguish in term of their morphological characters, mitochondrial genes and a single nuclear gene (Thai et al., 2007). Cyprinine is the one contains barbell so called Barbine while leuciscine is the one lacking of barbell. Each of these groups has its subfamilies in which Barbine consists of three subfamilies (Cyprininae, Gobioninae, and Rasborinae) and Leuciscini consists of four subfamilies (Acheiloganathinae. Cultrinae, Alburninae, and Leuciscinae) respectively (Briolay, 1998; Wang et al., 2012). One of examples in Cyprininae family is *Danio rerio* which is consider famous and the most commonly used model organism in vertebrate development biology.

2.5.1 *Rasbora sarawakensis*

Rasbora sawarakensis is a local species freshwater fish with a common name of Sarawak Rasbora. The genus *Rasbora* was first described by Bleeker in 1859 according to the previous study found by Kottelat (1999). Meanwhile, Brittan was continuing the study to elevate the species in the genus Rasbora in the 1950s (Siebert & Guiry, 1996) for examples species of *R. hubbsi* and *R. sarawakensis*.

R. sarawakensis are found in the island of Borneo especially in Sarawak, Malaysia. It is endemic to the island of Borneo and also distributed into a neighboring country such as Kalimantan Barat in Indonesia. In Sarawak, *R. sarawakensis* can be found in the slow-moving river water with thick marginal vegetation and in the stream that usually covered with the dense rainforest canopy to protect the river from heat and sunlight.

Batang Kayan (Figure 2.5) and Sungai Sarawak are the examples of habitat in Sarawak but also in the Mempawah and Melawi in Kalimatan Barat.

R. sarawakensis has orange fins, silver and electric blue midline (Figure 2.6) display on the body surface which attracted the attention of fish keeper. It has a relatively small body size with approximately about 3 to 4 cm long. The maximum standard length can go until 5 cm in this type of species. It belongs to the family of the Cyprinidae and subfamily of Danioninae in the class of ray-finned fishes (Surhone et al., 2011). This species usually makes a great school with 8 to 10 members in the planted aquarium so that they can display their outlook naturally especially for males to display their best colors when they are competing for female attention (*Rasbora sarawakensis*, 2012). For females, they are slightly larger and rounder than the males in term of sexual dimorphism. In the term of breeding of Rasbora species, it can be difficult because they lay their eggs under a rock or other hard items in the tank.

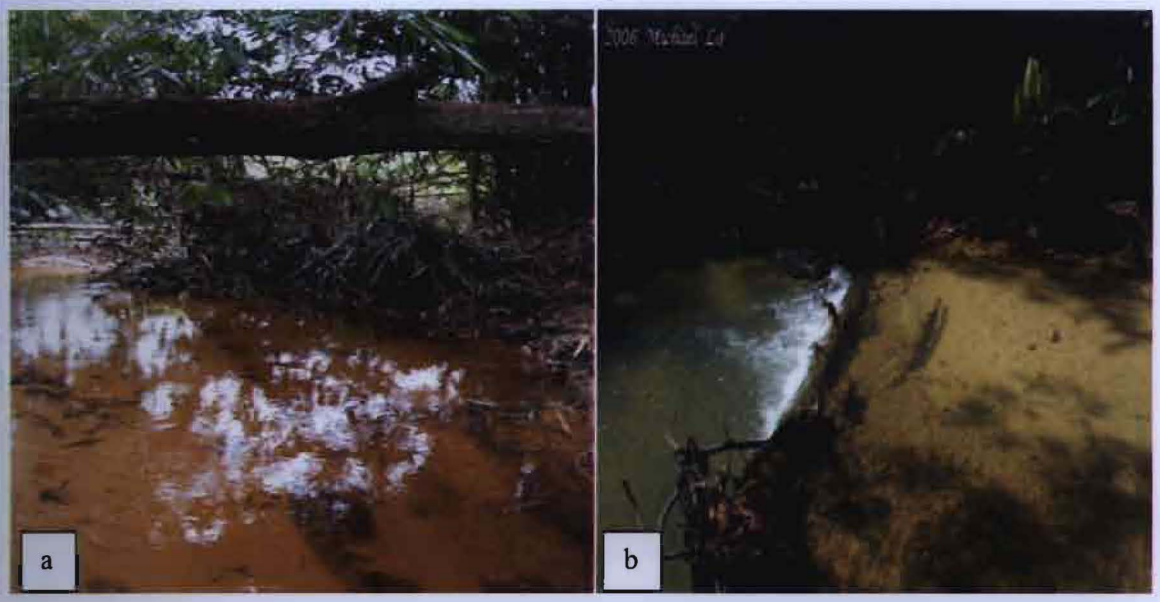

Figure 2.5 Habitat of *Rasbora sarawakensis* at: a) Batang Kayan, b) Headwater of the Sungai Sarawak. (Adapted from *Rasbora sarawakensis*, 2012)

Figure 2.6 *Rasbora sarawakensis* from Sungai Sebat, Western Sarawak, Borneo. (Adapted from *Rasbora sarawakensis*, 2012)